Micronucleus test in peripheral blood of rats treated with hyperbaric oxygen after subtotal splenectomy preserving the lower pole

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ABSTRACT

PURPOSE: To assess the mutagenic potential of the oxygen inhalation therapy (HBO), by means of the micronucleus test, performed in peripheral blood of rats that underwent subtotal splenectomy with lower pole preservation (ESTPI), after HBO sessions or simulations.

METHODS: Eighteen male Wistar rats, were distributed into three groups of six animals: group 1 - submitted to ESTPI and HBO sessions; group 2 - submitted to ESTPI and HBO simulations; group 3 – underwent cyclophosphamide administration. In groups 1 and 2, blood samples from the animals’ tails were collected before surgery (T0) and immediately after the 13th HBO session or simulation (T1). In group 3, tail blood samples were collected from animals before (T0) and 24 hours after (T1) cyclophosphamide (CP) delivery. The number of micronucleated normochromatic erythrocytes (MNNCE) was determined by blind counting 2000 normochromatic erythrocytes (NCE) per animal.

RESULTS: Micronuclei average after CP delivery in group 3 was higher than before its use, thus confirming the mutagenic activity of this drug (p=0.01). In groups 1 and 2, no significant difference in the average of Micronuclei was observed when comparing it to blood samples before and after the 13th HBO session or simulation.

CONCLUSION: The treatment protocol used in this study did not induce Micronucleus formation in animals submitted to ESTPI and HBO treatment or simulation.

Key words: Splenectomy. Mutagenicity Tests. Oxygen Inhalation Therapy. Rats.
Introduction

Hyperbaric oxygen therapy (HBO) is the exposure to oxygen inside a chamber under high pressure. The therapy aims at increasing the concentration of gas free fraction, which, when is not linked to haemoglobin and dissolved in plasma, reaches several body tissues. The protocol usually indicates the use of two absolute atmospheres of pressure, in sessions that last about 60 minutes. The therapy is indicated for several situations, including decompression sickness, acute carbon monoxide poisoning, air embolism, soft tissue infections and in treating delayed healing wounds.

Recently, other benefits from HBO were observed in experimental conditions. HBO treatment of the rats submitted to subtotal splenectomy with lower pole preservation (ESTPI) enabled the improvements in the lipid function and in the viability of the lower pole when compared with rats not submitted to HBO, as well as the increase in cellular and vascular proliferation. For the other hand, HBO has side effects such as pulmonary and neurologic toxicities, auditory barotrauma, discomfort in facial sinuses and temporary visual impairment. Such effects might be related to variations in the pressure and/or to the toxicity of oxygen, which in turn depends on its concentration when delivered during HBO.

The exposure to high concentrations of oxygen leads to increases on the number of free radicals and reactive metabolites, which might be the responsible for the toxic effects from therapy, since several reactive oxygen species might act directly in the DNA. As the occurrence of mutations might be related to carcinogenic events and cellular aging, studies evaluating HBO’s mutagenic capacity are of fundamental importance, since the therapy is being increasingly used. An easy way to identify in vivo chromosomal aberrations is the micronucleus test, which is considered as the gold standard method for cytogenetic analysis.

The micronucleus (MN), also known as Howell-Jolly corpuscle, is a small chromatin mass, separated from the main cellular nucleus, formed during the telophase in mitosis or meiosis. The MN results from acentric chromosome fragments or from extruding whole chromosomes from the main nucleus. When bone marrow erythroblasts expel their nuclei and become erythrocytes, the MN remains in the cytoplasm, where they are identified due to particular characteristics. The young erythrocytes called polychromatic, turn normochromatic (NCE) and get into the bloodstream. The MN can be spontaneously created or induced.

Some studies analyzed HBO mutagenic potential and divergent results were shown. Due to the important role in micronucleated cells splenic capture and since little is known about the impact of HBO on MN frequency on partially splenectomized animals, we evaluate the mutagenic potential of a HBO protocol by means of a micronucleus test performed in the peripheral blood of Rattus norvegicus Wistar rats submitted to ESTPI, according to techniques already described.

Methods

The present work is an experimental prospective study approved by the Ethics Committee on Animal Use of EMESCAM (protocol number 006/2011).

Sample characteristics and animal handling

Eighteen male Wistar rats (Rattus Norvegicus Albinus, Rodentia, Mammalia), 2-3 months old and weighing about 316g were used in the experiment. They were bred at the Research Center at EMESCAM, and kept in suitable and properly identified cages, under appropriate conditions to the species. The animals were divided into three groups with six animals each: group 1 - animals that underwent ESTPI and HBO; group 2 - animals that underwent ESTPI and HBO simulation; group 3 - animals submitted delivery of CP.

Anesthesia and subtotal splenectomy preserving the lower pole

Groups 1 and 2 were weighed and anesthetized with ketamine at 75 mg/kg of body weight associated with xylazine at a dose of 5 mg/kg, intraperitoneally delivered. Then the rats were immobilized on a surgical table for performing the thoracic and abdominal wall trichotomy as well as the abdominal wall antisepsis with a 10% povidone-iodine alcoholic solution.

The surgical procedure consisted of a median longitudinal incision in the skin and in the subcutaneous tissue of about 2.5 cm long and 0.5 cm below the xiphoid process. Then, the linea alba and peritoneum were opened. The spleen was mobilized to the surface of the abdominal cavity. The ligation and section of vessels that irrigate the upper and middle portion of the spleen were performed. It was done near to the splenic surface with nylon 6.0. The spleen was sectioned under lacquered vessels and the inferior pole was kept irrigated by gastrosplenic ligament vessels. The raw area of the lower pole was not sutured. The closure of the abdominal cavity was done by continuous sutureting the peritoneum and the musculoaponeurotic plan together and followed by the skin using mononylon 6.0.
After the surgical procedures, 5 ml of 0.9% saline were subcutaneously delivered for electrolyte replacement. Analgesia was done by orally dipyrone, dissolved in drinking water at a dose of 52.5 mg/day, for 72 hours, and nalbuphine hydrochloride at a dose of 0.1 mg/kg, subcutaneously delivered every 12 hours, during three days. Food and water ad libitum was offered after surgery.

**Oxygen inhalation therapy**

HBO was performed only in group 1, according to the previously established protocol. After the anesthetic effect, animals were placed inside the hyperbaric chamber and subjected to gradual compression of 2.5 atm, with 1 atm at sea level and 1.5 atm on the gauge camera for a period of 15 minutes. They were kept under such condition for 90 minutes, followed by exposition to gradual decompression chamber for 15 minutes. This procedure was performed twice a day, with three hours interval between sessions during the first three days and once a day on the following seven days. Group 2 was kept inside the hyperbaric chamber, simulating the same conditions set to the treated animals for identical time applied to group 1. All animals received 4 ml of 0.9% saline subcutaneously delivered after the 2nd HBO session or simulation in the first three days and after each session or simulation on the following seven days.

**Cyclophosphamide delivery**

A single 50mg/kg CP dose was administered in animals of group 3, intraperitoneally, according standard protocols. Tail blood samples were taken at the following times: T0 - before CP delivery and T1 - 24 hours after drug delivery.

**Micronucleus test in peripheral blood of rats**

Tail blood samples from all rats were collected at the following times: T0 - before surgery (groups 1 and 2) and before CP delivery (group 3); T1 - immediately after the 13th HBO session or simulation and 24 hours after CP delivery.

Smear was performed from peripheral blood samples, totaling two slides for each animal. They were fixed with methanol PA for ten minutes and after 24 hours they were stained with Leishman, using a modified Melo’s protocol: the slides were covered with pure Leishman stain for three minutes. They were then covered with Leishman solution in distilled water (1:6) for 15 minutes. Next, the slides were washed five times with pure distilled water.

**Animal euthanasia**

Animals from groups 1 and 2 were euthanized immediately after the last HBO session or simulation. Animals from group 3 were euthanized 24 hours after the CP delivery. Anesthesia was performed in order to collect the inferior pole of the spleen; a material that will be used in future studies. The used drugs were ketamine hydrochloride at a dose of 75 mg/kg of body weight associated with xylazine at a dose of 5 mg/kg, intraperitoneally. Euthanasia was performed with an overdose of sodium pentobarbital at a dose of 120 mg/kg intraperitoneally, and 10% potassium chloride, by means of an intracardiac injection (dose effect).

**Slides analysis**

The number of MNNCE was determined by the blind counting of 2000 NCE per animal at each time, totaling 24000 cells, per group. The counting was done in an Olympus® optical microscope (100X), under immersion.

**Variables and statistical tests**

After cytological analysis of slides containing samples of peripheral blood and checking normal probability distribution (Kolmogorov-Smirnov test), Student’s t test for paired samples was used, with a significance level of p<0.05, to study the presence or absence of MNNCE and their number in T0 and T1.

**Results**

Results are presented on Table 1. No animal had complications or died.

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**TABLE 1** Evaluation of micronucleus in animals of groups 1, 2 and 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Arithmetic mean</th>
<th>Standard deviation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T0</td>
<td>0.50</td>
<td>0.84</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>0.17</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>T0</td>
<td>0.33</td>
<td>0.52</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>T0</td>
<td>2.83</td>
<td>1.47</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>4.33</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>

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Discussion

With the purpose of verify the mutagenic effect of HBO treatment, in this study the presence of MN was analyzed in the peripheral blood of Wistar rats submitted to ESTPI followed by HBO treatment and, the increase of MN was not observed. Moreover, it was observed that the animals that underwent simulation also did not show increase in the number of MN, which might mean that the stress of this situation by itself is not mutagenic. In the group subjected to the application of CP, there was a significant formation of MN after 24 hours, demonstrating that it was possible to cause mutations and view them in peripheral blood cells, which is compatible with others studies. Another important variable in the micronucleus test is the sex, with males generally being more sensitive to the mutagenic agents than females to the induction of micronuclei. To avoid this problem, we studied only male rats.

Some studies analyzed HBO mutagenic potential and showed no significant MN increase both in humans and animals. However, divergent results were shown in cell cultures and human samples. Chromosomal aberrations were found in patients treated with HBO that had comorbidities and used different drugs. This study was conducted in ten sessions, with volunteers that were kept in a hyperbaric chamber with 100% oxygen at a pressure of 1.5 to 2.0 atm, for 40 minutes, with 15 minutes of compression and decompression. In another study, MN were also observed in cultures of V79 cells from Chinese hamster lung, widely used in genetic toxicology. The significant MN increase was observed after submitting the samples to 98% oxygen, at a pressure of about 3 atm for three hours. However, at lower exposure time, MN induction showed no significance.

A new study evaluated the effect genotoxic of HBO in peripheral blood samples from healthy volunteers by using the MN test and the Comet assay, which is another form of cytogenetic analysis. These individuals were treated with 100% oxygen at 2.5 atm pressure for three periods of 20 minutes, interspersed with five minutes of breathing room air. The samples were collected immediately, two and 24 hours after the therapy. The MN test did not show chromosomal damage in any of the analyzed periods. However, the Comet assay have detected primary DNA damage immediately after HBO, but not after two and 24 hours. The authors assumed that such result is due to a primary DNA repair mechanism. In a later study, the same group showed that a single HBO session induced adaptive response that protected human blood cells from the genotoxic effects caused by hydrogen peroxide. The great variety of HBO protocols described may confirm differences in presence of MN founded in many studies.

Reinforcing results from other studies, the current one showed no significant increase in the number of MN in animals treated with HBO. However, the animals used in this study were submitted to ESTPI, which may have influenced the number of MN found. Since it has been proven that there is an increase in the number of MN by the use of mutagens in rats submitted to total splenectomy, we propose that ESTPI would only partially decrease the splenic capture of these cells. Therefore, it is necessary to develop experiments using HBO in rats subjected to total and partial splenectomy as well as the preservation of the entire spleen. Studies using mutagens in rats undergoing ESTPI to compare the number of MNCE in each of these cases are also needed, thus they may allow establishing the most precise way to safely apply the therapy associated with conservative spleen surgery procedures.

Conclusion

The HBO protocol used in this study did not induce mutagenicity in rats submitted to subtotal splenectomy preserving the lower pole of the spleen. However, specific definitions about the ideal pressure or duration of the HBO sessions were not established.

References


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